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Short Communication

Differential diagnosis and molecular characterization of *Theileria* spp. in sika deer (*Cervus nippon*) in Hokkaido, Japan

Seung-Hun Lee[a], Paul Franck Adjou Moumouni[a], Eloiza May Galon[a], Patrick Vudriko[a], Mingming Liu[a], Byamukama Benedicto[a], Maria Agnes Tumwebaze[a], Damdinsuren Boldbaatar[b], Rika Umemiya-Shirafuji[a], Shinya Fukumoto[a,*], Xuenan Xuan[a,*]

[a] National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan
[b] Institute of Veterinary Medicine, Zaisan 17024, Ulaanbaatar, Mongolia

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ABSTRACT

Sika deer (*Cervus nippon*) is widely distributed in Asian countries and is one of the most common wildlife animals in Hokkaido, Japan. Previous studies identified *Theileria* spp. in sika deer in Japan including *Theileria* sp. Thrivae belonging to *T. cervi* group and *Theileria* sp. sola belonging to *T. capreoli* group. However, the studies failed to differentiate these two species without sequencing. Therefore, epidemiological information on cervine theileriosis in Hokkaido, Japan is limited. This study differentiated the two *Theileria* spp. using restriction fragments length polymorphism (RFLP). Based on the PCR-RFLP, *Theileria* spp. were identified in 103 (88.0%) of 117 samples, and the prevalence of each parasites were 86.3% (n = 101) and 57.3% (n = 67) for *Theileria* sp. Thrivae and *T. capreoli*-like, respectively. Phylogenetic analysis based on the 18S rRNA showed a close relationship between *Theileria* sp. Thrivae and *T. cervi* in China. In addition, phylogenetic analysis of internal transcribed spacer regions also showed a close relationship between *Theileria* sp. Thrivae and *T. cervi*.

Sika deer (*Cervus nippon*) is distributed in Asian countries and one of the most common wildlife animals in Hokkaido, Japan [1]. Recently, increased number of sika deer caused problems not only in agriculture and forestry but also in tick-borne pathogens [1,2]. Because sika deer are responsible for different tick species including *Haemaphysalis longicornis*, *H. yeni*, *H. flava*, *H. megaspinosa*, *H. kitaokai*, *Ixodes ovatus*, and *Amblyomma testudinarium* [3], increasing number of sika deer suggests possibility of spreading of tick-borne diseases [1,4].

Theileria spp. which belong to the phylum Apicomplexa are tick-borne hemoprotozoan parasites that infect various domestic and wildlife animals [5]. Depending on the infectious agent and host characteristics, clinical symptoms vary from fatal to subclinical and cause economic burden on livestock industry [6]. Among *Theileria* spp., *T. cervi*, *T. capreoli*, *T. uilenbergi,* and *T. luwenshuni* are known to cause cervine theileriosis [7–9].

Different studies previously identified *Theileria* spp. in sika deer in Japan [1,4,10,11]. Ever since Inokuma et al. [10] first identified *Theileria* sp. Thrivae (synonym *Theileria* sp. sika 1) which is closely related to *T. cervi*, Watanabe et al. [4] recently revealed *Theileria* sp. Sola which belongs to *T. capreoli* group (hereafter, *T. capreoli*-like). Because Watanabe et al. [4] confirmed the species based on sequence analysis, the method has limitation to be applied for broad epidemiological study. Recently, a few studies differentiated *Theileria* sp. Thrivae from *Theileria* spp. using PCR based assays, although other *Theileria* spp. were not evaluated [1,12]. Differential diagnosis of a pathogen is the most principal and essential part for evaluation, treatment, and prevention of disease. Therefore, a simple method needs to be developed to evaluate prevalence of each *Theileria* spp. in sika deer.

The purpose of this study was a twofold. First, to evaluate prevalence of *Theileria* sp. Thrivae and *T. capreoli*-like in sika deer in Hokkaido, Japan. Secondly, molecular characterization of the two *Theileria* spp. using phylogenetic analysis based on the 18S rRNA and ITS regions.

This study was approved by Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido, Japan (Permission number: T28–39).

One hundred seventeen sika deer blood was collected from April 2010 to March 2011 from 8 different towns (Erimo, Hiroo, Obihiro, Samani, Onbetsu, Hidaka, Urakawa, Urahoro, and Toyokoro) in Hokkaido, Japan (Fig. S1). Additionally, information on the sex of sika

* Corresponding authors at: National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan.
E-mail addresses: fukumoto@obihiro.ac.jp (S. Fukumoto), gen@obihiro.ac.jp (X. Xuan).

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deer was collected.

DNA from sika deer blood was extracted using DNA extractor® WB Kit (Wako, Tokyo, Japan) according to the manufacturer's instruction. The extracted DNA was stored at −30 °C until further experiments.

PCR was performed using TaKaRa Ex *Taq*® DNA polymerase (Takara, Shiga, Japan) according to manufacturer's recommendations. Specific primers targeting 18S rRNA of *Theileria* genus were used to detect *Theileria* spp., as previously described [13]. For the positive control, the first identified *Theileria* sp. Thrivae-positive sample was used after cloning into *Escherichia coli* DH5α, while distilled water was used as negative control.

To differentiate *Theileria* sp. Thrivae and *T. capreoli*-like, two restriction enzymes named *Psi*I (New England Biolabs, Ipswich, MA, USA) and *Psh*AI (New England Biolabs) were selected for RFLP analysis. Reactions were done according to the manufacturer's recommendation. The expected PCR-RFLP patterns are 689, 393, and 25-bp for *T. capreoli*-like with *Psi*I and no-cut for *T. capreoli*-like with *Psh*AI. Meanwhile, 1077 and 25-bp for *Theileria* sp. Thrivae with *Psi*I, and 737 and 365-bp for *Theileria* sp. Thrivae with *Psh*AI.

Based on the PCR, *Theileria* spp. were identified in 103 (88.0%) of 117 samples (Table S1). The PCR-RFLP clearly distinguished the two *Theileria* spp. (Fig. 1), consequently, 101 (86.3%) and 67 (57.3%) positive samples for *Theileria* sp. Thrivae and *T. capreoli*-like, respectively, were identified. All the *T. capreoli*-like infected samples were co-infected with *Theileria* sp. Thrivae.

Association between prevalence and sex of sika deer was evaluated by Chi-square test using R [14]. *P*-value < .05 was considered as statistically significant. However, there was no statistically significant difference regarding *Theileria* spp., *T. capreoli*-like, and *Theileria* sp. Thrivae ($P > .05$). The samples ($n = 15$) without data on sex were excluded for statistical analysis.

After screening of *Theileria* spp., five *Theileria* sp. Thrivae and four *T. capreoli*-like PCR positive samples were submitted for sequencing using *E. coli* DH5α and pGEM-T easy vector system (Promega, Madison, WI, USA). At least three positive colonies from each sample were selected. Using a universal primer set (M13/pUC forward and reverse), cycle sequencing was performed with BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the results were analyzed by ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Based on the *Theileria* genus specific primers targeting 18S rRNA, 1102-bp and 1107-bp for *Theileria* sp. Thrivae and *T. capreoli*-like, respectively, sequences were obtained. The two *Theileria* spp. showed 97.9% genetic identity each other.

In addition to the identification of 18S rRNA of *Theileria* spp., ITS regions (ITS-1 and ITS-2) and 5.8S rRNA of *Theileria* spp. were also amplified and analyzed for molecular characterization, as previously described [6]. In case of *Theileria* sp. Thrivae, 985-bp to 992-bp of ITS

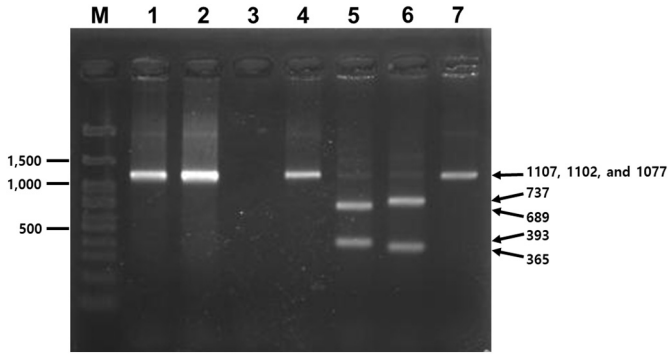

Fig. 1. PCR-RFLP results of *Theileria* sp. Thrivae and *T. capreoli*-like visualized by gel electrophoresis. PCR product size are indicated on the right. M, 100 bp marker; 1, *T. capreoli*-like; 2, *Theileria* sp. Thrivae; 3, PCR negative control; 4, *T. capreoli*-like treated by *Psh*AI; 5, *T. capreoli*-like by *Psi*I; 6, *Theileria* sp. Thrivae by *Psh*AI; 7, *Theileria* sp. Thrivae by *Psi*I.

regions and 5.8S rRNA were obtained. The percent identity values of ITS regions showed that there was a high genetic diversity of this region among *Theileria* spp. (Table S2). However, the ITS regions of *T. capreoli*-like were not identified by cloning and sequencing.

Some representative sequences identified in this study were submitted to GenBank database (18S rRNA of *T. capreoli*-like and *Theileria* sp. Thrivae, MH489003 – MH489011; ITS regions of *Theileria* sp. Thrivae, MH491837 – MH491841).

Phylogenetic tree was constructed based on *Theileria* spp. 18S rRNA and ITS regions using MEGA 7.0 with maximum likelihood method [15]. Topology of the tree was supported by 500 bootstrap replications. Data regarding host species and isolated countries were included in the tree. As outgroup, *Plasmodium falciparum* (XR_002273101) and *P. vivax* (AF316893) were included for 18S rRNA and ITS regions, respectively.

Phylogenetic analysis based on the 18S rRNA clearly differentiated *T. capreoli*-like and *Theileria* sp. Thrivae (Fig. 2A). In addition, *Theileria* sp. Thrivae clustered with *T. cervi* which was identified in China (KT959224) and *T. capreoli*-like clustered with *T. capreoli* from China and Spain (KJ188219 and AY726011). However, *T. cervi* identified from USA (U97054 – U97056) showed clearly different clade with *T. cervi* from China. Based on the phylogenetic analysis of *Theileria* spp. ITS regions, *Theileria* sp. Thrivae showed close relationship with *T. cervi* in China (Fig. 2B).

This study differentiated the two *Theileria* spp. using PCR-RFLP and the results revealed high prevalence not only in *Theileria* sp. Thrivae but also in *T. capreoli*-like in sika deer in Hokkaido, Japan. In case of *Theileria* sp. Thrivae, high prevalence in this study is consistent with the previous studies such as 87.7% (236/269) [1] and 69.4% (25/36) [4]. However, prevalence of *T. capreoli*-like in this study was obviously higher than the previous study (2.8%, 1/36) [4].

Despite of high prevalence of *Theileria* spp. in sika deer in Japan, pathogenicity of the two *Theileria* spp. remains unclear [4]. Previous studies indicated that cervine theileriosis are generally considered benign, although, it can cause clinical symptoms under stressful conditions like overcrowding, poor nutrition, and secondary infection [8,16]. Considering their natural habitat, sika deer live in the wild as a colony and hardly express clinical symptoms except for fatal cases, and it is difficult to characterize pathogenicity of the two *Theileria* spp. [17]. Therefore, profound study on pathogenicity of the two *Theileria* spp. in a controlled environment are still warranting.

Because deer are a host of different tick-borne pathogens, previous studies investigated possible role of deer in transmission of tick-borne pathogens between deer and other animals. In cases of *Babesia*, *B. bovis* and *B. bigemina* were identified in deer [18,19], on the other hand, bovine *Theileria* has not been identified in deer [5]. A recent study investigated possible transmission of *Theileria* sp. Thrivae between sika deer and cattle [12]. However, *Theileria* sp. Thrivae was identified only in sika deer and ticks including *I. persulcatus* and *H. japonica*, not in cattle, which suggest host specific characteristics of *Theileria* sp. Thrivae.

Phylogenetic analysis based on the 18S rRNA showed clustering of *T. capreoli*-like with *T. capreoli* and *Theileria* sp. Thrivae with *T. cervi* which is consistent with the previous study [4]. Additionally, phylogenetic analysis based on the ITS regions also showed close relationship of *Theileria* sp. Thrivae with *T. cervi*. Based on these results we believe that *Theileria* sp. Thrivae is identical species with *T. cervi* which was identified in China. However, since *T. cervi* in USA which showed clearly different phylogenetical relationship, its taxonomical classification need to be clarified.

Sequence analysis of ITS regions revealed a high genetic diversity of ITS-1 and ITS-2 regions, and conserved characteristics of 5.8S rRNA among *Theileria* spp. (Tables S3–S5). These results are consistent with the previous studies that showed genetic diversity of ITS regions in *Theileria* spp. [20]. It is worth noting that, in cases of ITS-1 and ITS-2, they showed highly diverse characteristics even in same species from same country (HQ184413 and HQ184417). High genetic diversity of

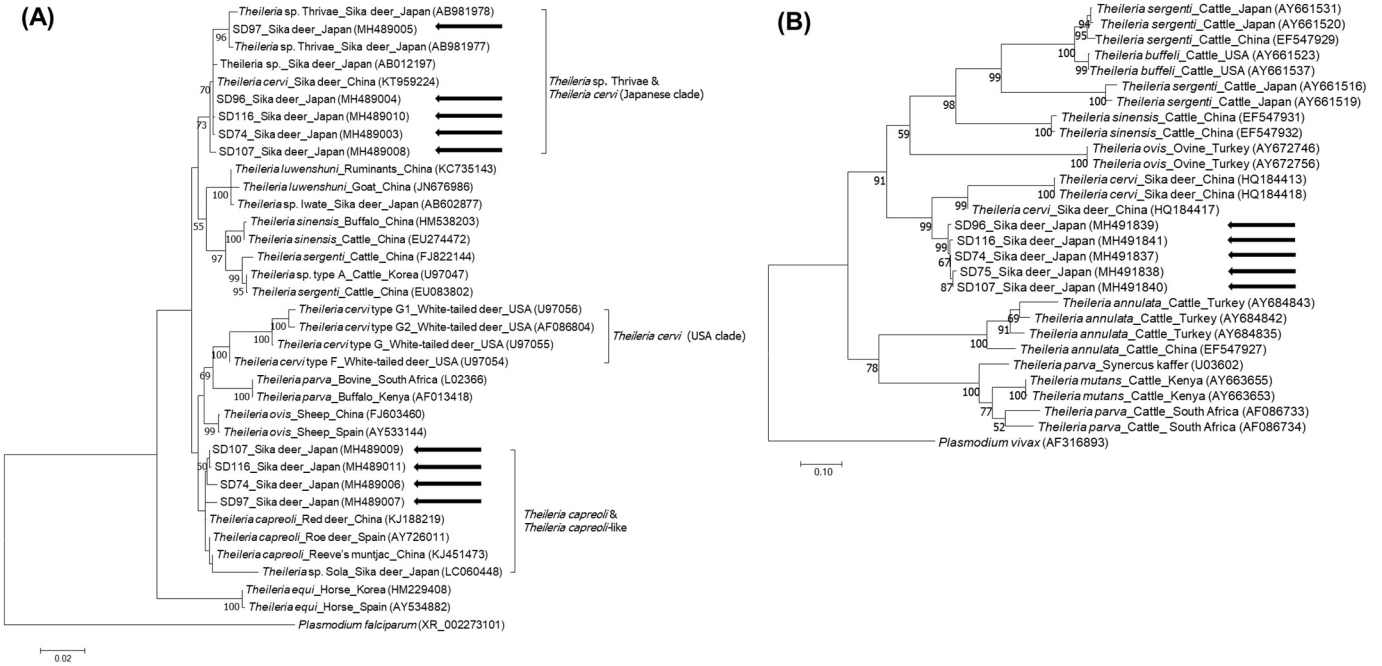

Fig. 2. Phylogenetic analysis of *Theileria* spp. based on (A) 18S rRNA and (B) ITS-1 region. The phylogenetic trees were constructed by maximum-likelihood method with 500 bootstrap replications. Species, host, country, and GenBank accession number are described in the sequences. Bootstrap values < 50 were omitted.

ITS-regions suggests the usefulness of this region as a molecular marker to differentiate *Theileria* spp.

In the present study, only ITS regions' sequence from *Theileria* sp. Thrivae was obtained using cloning with *Theileria* genus specific primers, whereas no positive clones of *T. capreoli*-like were obtained. It could be attributed to two factors. The first factor is genetic diversity of ITS regions of *T. capreoli*-like. Although this study used *Theileria* genus specific primers to amplify ITS regions [6], the possibility of genetic diversity of primer annealing parts of *T. capreoli*-like could not be excluded which resulted in unsuccessful PCR. In addition, ITS regions' sequence of *T. capreoli* also has not been identified yet. The second reason is the low DNA concentration of *T. capreoli*-like. In this study, amplicons of *Theileria* spp. 18S rRNA inserted into *E. coli* DH5α were confirmed by sequencing and only a few clones were confirmed as *T. capreoli*-like and majority of the clones contained sequences from *Theileria* sp. Thrivae. This result suggests low DNA concentration of *T. capreoli*-like than that of *Theileria* sp. Thrivae in tested samples.

In conclusion, this study developed a simple assay to differentiate *T. capreoli*-like and *Theileria* sp. Thrivae using PCR-RFLP which can be applied for broad epidemiological study. This study revealed high prevalence of *T. capreoli*-like suggesting potential importance to cervine theileriosis in Hokkaido, Japan. In addition, phylogenetic analysis based on the 18S rRNA and ITS regions showed a close relationship between *Theileria* sp. Thrivae and *T. cervi*.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parint.2019.01.005.

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